2. MATERIALS AND METHODS

2.1. Isolation of 7.5 dpc to 17.5 dpc mouse embryos

Reagents and Solutions

1X Phosphate Buffered Saline (PBS)
NaCl (USB, SC, USA) 8.0 g
KCl (Fisher-Scientific, CO, USA) 0.2 g
Na$_2$HPO$_4$ (USB) 1.44 g
KH$_2$PO$_4$ (USB) 0.24 g

These chemicals were dissolved in 800 ml deionized water and pH was adjusted to 7.4 with 1 N HCl (Thermo-Scientific). The volume was made up to 1000 ml. The solution was stored at 4°C.

4% Para-formaldehyde Solution (PFA)
Para-formaldehyde powder (Sigma Aldrich, MO, USA) 4 g
1X PBS 80 ml

Reagents were dissolved and pH was adjusted to 7.4 using 5M NaOH. Volume was made up to 100 ml. Solution was autoclaved for sterilization and aliquoted in 2 ml tubes. Aliquots were stored in -20°C.

Trizol
Trizol was commercially procured from Invitrogen NY, USA.

Methodology
Swiss Webster (CFW®) male mice (6-8 weeks old) were mated with females (4-6 weeks old) over-night. The next morning after mating, the females were checked for the presence of a copulation plug in the vagina. Presence of vaginal plug indicated that mating occurred; however it didn’t ensure pregnancy. Using conventional approach, at noon of the next day (the day on which the vaginal plug was found) of mating, the age of embryos was considered to be 0.5 dpc (days post coitum). Plugs were always checked early in the morning because they often fall out or no longer detectable ~12 hours after mating. Embryos ranging from 7.5 dpc to 17.5 dpc were isolated by dissecting impregnated mice using dissection microscope and instruments. Uterus of pregnant mice was washed with 1 XPBS. Uterus of 7.5 dpc to 17.5 dpc impregnated female mice
appeared like beads in a string. Using fine forceps, muscular wall of uterus was separated. Embryos were isolated by removing the Reichert's membrane and visceral yolk sac surrounding them. These embryos were very carefully washed with 1X PBS. Half the embryos were fixed in 4% PFA for in situ hybridization and remaining embryos were stored in Trizol at -80°C until use. The embryos within a uterus varied with regard to stage of development. Therefore, morphological criteria were used to identify precise embryo staging using “Theiler Staging Criteria for Mouse Embryonic Development”.

2.2. Maintenance of cell lines

Reagents and Solutions

Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (4.5g/L):
DMEM Powder (Invitrogen,) 4.5g
Glutamine (Invitrogen) 0.3 g
Sodium Bicarbonate (Sigma Aldrich) 3.7 g
Reagents were dissolved in 800ml of deionized water and pH was adjusted to 7.4 by bubbling CO₂ gas. The volume was made up to 1 L. Media was filter sterilized using a 0.22 μm filter and stored at 4°C.

Minimum Essential Medium, Earle’s (MEM-E):
MEM-E Powder (Invitrogen,) 9.53 g
Glutamine 0.3 g
Sodium Bicarbonate 2.2 g
Reagents were dissolved in 800ml of deionized water and pH was adjusted to 7.4 by bubbling CO₂ gas. The volume was made up to 1 L. Media was filter sterilized using a 0.22 μm filter and stored at 4°C.

Antibiotics stock, P&S (100X):
Penicillin (Sigma Aldrich) 1X10⁶ units
Streptomycin (Sigma Aldrich) 10 g
Reagents were dissolved in 100 ml tissue culture grade water and solution was filter sterilized using a 0.22 μm filter. 1 ml aliquots were made and stored at -20°C till use.

TPVG:
0.1% Trypsin (Invitrogen)
0.02% EDTA (Versene) (Invitrogen)
0.05% glucose (Invitrogen)
Reagents were dissolved in 1X PBS, with phenol red as pH indicator (1 ml of 1% stock). pH was adjusted to 7.4, filter sterilized and stored at 4ºC.

**1X Phosphate Buffered Saline (PBS)**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.44g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.24 g</td>
</tr>
</tbody>
</table>

These chemicals were dissolved in 800 ml deionized water and pH was adjusted to 7.4 with 1 N HCl. The volume was made up to 1000 ml. The solution was filter sterilized using 0.22 µm filter and was stored at 4ºC for future use.

**Methodology**

NIH/3T3 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma) supplemented with 10% bovine serum (Invitrogen), 1X penicillin and streptomycin solution and 2 mM glutamine (Invitrogen) in a humidified incubator (Thermo Scientific, IL, USA) at 37ºC with 5% CO$_2$. The cells were passage after they reached 70-80% confluency by trypsinization using TPVG. SK-N-MC cells were cultured in Minimum Essential Medium (MEM) (Sigma) and supplemented with 10% fetal bovine serum (Invitrogen), 1X pencillin and streptomycin solution, 2mM of Sodium Pyruvate (Invitrogen) and 2 mM glutamine (Invitrogen) in a humidified atmosphere at 37ºC with 5% CO$_2$. The cells were passage after they reached 80-90% confluency by trypsinization using TPVG.

**2.3. Trypan Blue Exclusion Test for Cell Viability**

**Reagents and Solutions**

**0.4% Trypan Blue solution (Sigma Aldrich)**

**Methodology**

Cells were checked for viability by Trypan blue exclusion test. Cells were trypsinized and suspended in medium. Cell suspensions were mixed with trypan blue solution in 1:1 ratio and incubated for 3 min at room temperature. 10 µl of mixture was loaded on haemocytometer and unstained (viable) and stained
(nonviable) cells were counted separately. Percentage viability was calculated as follows:

\[
\text{Viable cells (\%)} = \frac{\text{Viable cells}}{\text{Total number of cells}} \times 100
\]

2.4. Mammalian Cell Transfection

Reagents and Solutions

Opti-MEM medium
Opti MEM Powder (Invitrogen)
- Glutamine: 0.3 g
- Sodium bicarbonate: 2.4 g

Reagents were dissolved in 800 ml of deionized water and pH was adjusted to 7.2-7.4 by bubbling CO2 gas. The volume was made up to 1 L, filter sterilized and at stored at 4ºC.

G418 sulphate (1 mg/ml)
5 mg G418 powder (Invitrogen) was dissolved in 5 ml of tissue culture grade water, filter sterilized using a 0.22 µm filter and aliquoted. Aliquots were stored in dark at -20ºC.

Methodology

Ginir and its antisense Giniras were cloned into pTargeT mammalian expression vector (Promega, WI, USA) by TA cloning strategy as shown in Fig 2.1. These plasmid constructs were transfected into NIH3T3 and SK-N-MC cells to understand their functionality in these cells. Cells transfected with Empty Vector served as controls for each cell-line. For transfection, cells were trypsinized and counted with the help of haemocytometer as described above. Cells \((3 \times 10^4)\) were seeded in 1 ml of media containing 10% FBS in 6 well tissue culture plate. After 16 h, cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Briefly, 2 µg of plasmid DNA and 2 µl of Lipofectamine 2000 was suspended in 200 µl each of Opti-MEM and incubated separately for 5 min. Both these solutions were mixed together and incubated further for 15 min. This entire mixture was added to cells containing 1 ml of medium. The cells were further incubated with this complex overnight in a humidified atmosphere at 37ºC. Later, transfection was terminated by removing the medium containing excess Lipofectamine 2000-DNA
complex and by adding fresh culture medium. These cells were allowed to grow for 24-48 h. Cells were then trypsinized and plated in 100 mm dishes. After 24 h, 700 μg of G418 was used for selecting the cells. After 2 weeks, 6 clones per transfection were picked up and propagated as independent cell lines in G418 for 2-3 passages. After establishing these clones, over-expression of Ginir and Giniras was tested using strand-specific PCR as described in section 2.11. Only those clones were chosen for study that showed Ginir and Giniras over-expression by >2 fold and were propagated as independent cell lines. We have performed these transfections thrice and from each transfection on an average 6 independent clones were chosen.

2.5. *In vivo* tumor formation assay

**Reagents and Solutions**

**1X Phosphate Buffered Saline (PBS)**
Solution was prepared as described in section 2.2.

**Minimum Essential Medium, Earle's (MEM-E):**
Solution was prepared as described in section 2.2.
Methodology

SK-N-MC-EV, SK-N-MC-Ginir and SK-N-MC-Giniras cells were counted using haemocytometer and 1 X10^6 cells were injected subcutaneously into NOD/SCID mice. At least 5 mice were taken in each group. Mice were observed for tumor development for up to eight weeks. After 8 weeks mice were sacrificed by cervical dislocation and tumors were dissected out. For histology, tumors were directly fixed in 10% formalin. For culturing tumor cells, a part of the tumor was excised surgically and placed immediately in vials containing DMEM with 5X antibiotic Penicillin and streptomycin (P&S). The sample was processed immediately after dissection. Initially, the tissue was treated sequentially with DMEM containing varying concentrations of antibiotics 20X, 10X and 2X for about 20 minutes. Later, the tissue was minced with scissors into small pieces of about 1 mm^3 size and the pieces were processed as both explant cultures and adherent monolayer cultures. For explant cultures, the tissue pieces ( explants) were placed in 55 mm dishes using fine forceps and a drop (~20 ul) of FCS was added over each explant and incubated at 37°C for 2 h. Later, plates were fed carefully with complete medium (DMEM +10% FCS) without disturbing explants and incubated at 37°C. The cultures were observed microscopically for cell growth from the tissue explants. The cells were fed twice a week. For establishing adherent cultures the tissue pieces were treated with TPVG at 37°C for 30 minutes and later spun down. The supernatant was seeded in a 35 mm dish and propogated in MEM (E) + 10% FCS medium.

2.6. Spheroid formation assay

Reagents and Solutions

Minimum Essential Medium, Earle’s (MEM-E): Media was prepared as described in section 2.2.

Methodology

SK-N-MC-EV, SK-N-MC-Ginir and SK-N-MC-Giniras cells were trypsinized and counted for plating. 1 X 10^4 cells were plated in a 100 mm low attachment dishes for sphere formation in MEM-(E) + 10% KnockOut Serum Replacement (KOSR) (Invitrogen) medium. Medium of the plates was changed alternate day. Sphere formation was seen after 6-7 days. For secondary sphere formation assay, 0.3 X10^2-1.5 X10^2 cells per well were plated in low attachment 96 well plate in 80 µl medium. After every alternate day, 20 µl of fresh medium was added. Spheres
formed in each well after 6 days were manually counted and plotted as a bar graph to determine the sphere forming efficiency of SK-N-MC Ginir cells in comparison to SK-N-MC-EV and SK-N-MC-Giniras cells.

2.7. Cell proliferation assay

Materials and reagents

MTT Solution (5mg/ml)

50 mg MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was dissolved in 10 ml 1X PBS and then solution was sterilized by filtration using 0.22 μm syringe filter. 1 ml aliquots were prepared and stored at -20ºC until use.

Methodology

To determine the proliferation potential of NIH-EV, NIH-Ginir and NIH-Giniras cells, MTT assay was performed over an eight days period. The assay is based on ability of metabolically active cells to cleave yellow tetrazolium salt MTT to form dark blue formazan crystals. Metabolically active cells have active mitochondrial enzyme succinate dehydrogenase which catalyses the breakdown of MTT to form blue coloured formazan crystal. The formazan produced is solubilised in DMSO (Fisher Scientific) and the optical density (O.D.) is measured at 570 nm. The O.D. obtained is directly proportional to the number of proliferating cells in the assay. To perform this assay, equal number of NIH-EV, NIH-Ginir and NIH-Giniras cells were plates at a density of 1X 10³ cells per well in 96 well plate in triplicates for growth curve analysis. Cells were incubated in a humidified incubator at 37ºC, 5% CO₂. At every 24 h interval, 20 µl of 5 mg/ml MTT was added to the respective set of cells and the plate was incubated for 4 h at 37ºC, 5% CO2. The formazan crystals were dissolved in 200 µl of DMSO and the Absorbance was measured at 570 nm using ELISA reader. The experiment was performed in triplicates from two independent cell lines of each origin. For studying growth kinetics in SK-N-MC EV, SK-N-MC Ginir and SK-N-MC Giniras cells, same procedure was followed as described above for a period of 4 days.
2.8. Cell cycle kinetics

Materials and Reagents

1X PBS
Solution was prepared as described in section 2.1.

4% Para-formaldehyde Solution (PFA)
Solution was prepared as described in section 2.1

Propidium Iodide (Sigma Aldrich) solution (50 µg/ml)
1 mg of Propidium Iodide powder was weighed and dissolved in 20 ml of 1X PBS. Aliquots were prepared and stored at 4°C.

10mM Sodium Acetate solution pH 5.2
140 mg sodium acetate trihydrate (Fisher-scientific) was dissolved in 80 ml of deionized water and pH was adjusted to 5.2 using glacial acetic acid (Fisher-Scientific). Volume was made up to 100 ml with deionized water. Solution was autoclaved and kept at room temperature for further use.

1M Tris-HCl pH7.4
12.1 g Tris base (Sigma Aldrich) powder was dissolved in 40 ml of deionised water; pH was adjusted to 7.4 with conc. HCl. The volume was made up to 100 ml. The solution was stored at 4°C.

RNase A Solution (10 mg/ml)
RNase A powder (Sigma Aldrich) 0.5 g
10mM sodium acetate buffer (pH 5.2) 35 ml
Reagents were mixed and were heated at 100°C for 15 min in 10mM Sodium Acetate solution pH 5.2. Solution was allowed to cool at room temperature and pH was adjusted to 7.4 using 1 M Tris-HCl. RNase A solution was aliquoted to 1.5 ml tubes and kept at -20°C until use.

Methodology

Cells were plated at a density of approximately 1x10^5 cells in 60 mm dish and allowed to grow for 24 h. Cells were harvested by trypsinization and processed for flow-cytometry. Cells were washed with chilled PBS and fixed with
4% PFA. Cells were then permeablized using 0.1% Triton X-100 and given 
RNase treatment for 30 min at 37ºC. Cells were washed twice with 1X PBS and 
followed by addition of PI which was incubated in dark for 30 min on ice. The 
fluorescence of PI was collected through a 585 nm filter in flow cytometer (FACS 
Vantage, Becton Dickinson (BD Biosciences, CA, USA). The data were analyzed 
using cell quest software version (BD Biosciences) for 10,000 cells.

2.9. Real time PCRs
Reagents and Solutions

3M Sodium Acetate Solution (pH 5.2)
40.8 g sodium acetate trihydrate (Qualigens) was dissolved in 80 ml of deionized 
water and pH was adjusted to 5.2 using glacial acetic acid. Volume was made up 
to 100 ml with deionized water. Solution was autoclaved and kept at room 
temperature for further use.

RQ1 RNase-Free DNase (Promega)
Kit was procured from Promega and contained following components:
1) RQ1 RNase-Free DNase 1,000 U
2) Stop Solution 1.0 ml
3) RQ1 DNase 10X Reaction Buffer 1.0 ml

Reverse Transcription system
Kit was procured from Promega and contained following components:
1) AMV Reverse Transcriptase (High Conc.) 1,500 u
2) Recombinant RNasin® Ribonuclease Inhibitor (a) 2,500 u
3) Oligo(dT)15 Primer (0.5 µg/µl) 50 µg
4) Random Primers (0.5 µg/µl) 50 µg
5) dNTP Mixture, 10mM 320 µl
6) Reverse Transcription 10X Buffer 1.4 ml
7) MgCl2 25mM 1.2 ml
8) Nuclease-Free Water 13 ml

Sybr Green master mix
Sybr Green master mix was procured from (ABI, Invitrogen) and was stored at -
20ºC until use.


10mM Primer solution

Lyophilized primers were custom synthesized by Integrated DNA technologies (IDT) (Banglore, India).

Primer Stock Solution (100mM)

Primers were diluted to make a stock solution of 100mM using deionized water as per manufacturer’s instructions and kept in -20ºC.

Primer working solution

Primer stock solution was diluted in 1:10 ratio, which was kept in -20ºC for future use. Primers used for strand specific cDNA preparation and for real time are enlisted in Table 2.1

Methodology

For analysing expression of Ginir and Giniras in various cell lines and embryos, strand specific real time PCR was performed to investigate expression levels of Ginir and Giniras in various cell lines and embryos. 100 mm dish containing nearly 1X10^7 cells were rinsed with 1X PBS and 3 ml of Trizol (Invitrogen) was added to each plate. Plates were kept on a rocker for 5 min for lysis and 1 ml of the solution was collected into three 1.7 ml tubes containing 200 µl of Chloroform (Fisher Scientific). The tubes were inverted several times for a uniform suspension and left on ice for 10 minutes. Tubes were spun at 10,000 X g for 10 minutes and supernatant was collected into 600 µl of Isopropanol (Fisher Scientific). Tubes were kept in -80ºC for 10 minutes to overnight and spun at 10,000 X g for 20 minutes. Pellet was washed with 70% ethanol (Merck NJ,USA) twice at room temperature. Pellet was reconstituted in 50 µl of double distilled water or DEPC treated water and kept at 65ºC for 10 min. It was then quick chilled on ice for 2 min and was quantitated on nanodrop (Eppendorf, GmbH, Germany) and 15 µg of RNA was given DNase (Promega) treatment. For isolation of RNA from mouse embryos, appropriate amount of Trizol was added to the embryo depending on its size and was crushed using gentle MACS dissociator (Miltenyi biotec, GmbH, Germany). Freshly minced tissue was kept in Trizol on ice for 30 min and 1 ml of solution was collected in 1.5 ml tubes containing 200 µl of chloroform. After that, same procedure was followed as discussed above.
Reaction Mix:

RQ1 DNase 10X Reaction Buffer - 10 µl
RQ1 RNase-Free DNase (1U/µl) - 10 µl
RNA- 15 µg

Nuclease Free water (Promega) was added up to 100 µl.

Reaction was incubated at 37°C for 30 min and was stopped by adding 1 µl of stop solution for 15 min at 65 °C. RNA was again precipitated by adding 1/10 of total volume of 3M sodium acetate (pH 5.2) and 2 volumes of isopropanol (Qualigen). Tubes were kept in -80°C for at least 30 min. Tubes were spun at 4°C, 12,000 rpm for 15 min. RNA pellet obtained was washed with 70% ethanol (Merck,) and was reconstituted in 10 µl of NFW (Promega). 2 µg of RNA was used for making cDNA using Reverse Transcription system (Promega) in following reaction.

Table 2.1 List of Primers

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer</th>
<th>Sequence</th>
<th>Size of Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td>FP</td>
<td>AGTGTAGTGGCGGCGATTTC</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>CGAAATACCGAACACCTATAAT</td>
<td></td>
</tr>
<tr>
<td>p19</td>
<td>FP</td>
<td>ATGCTGCTGGAGGAGGTTTCA</td>
<td>242</td>
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<tr>
<td></td>
<td>RP</td>
<td>GTGCTGCAACACATCATGAC</td>
<td></td>
</tr>
<tr>
<td>Ginir( Real Time)</td>
<td>FP</td>
<td>ACCCAGAGACTGCCCTACCT</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>CCTGCAAGCCATTCTCTCAAC</td>
<td></td>
</tr>
<tr>
<td>Ginir( Strand Specifc cDNA)</td>
<td>FP</td>
<td>GGATATCTAACAACACCTGAAA AGC</td>
<td>557</td>
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<tr>
<td></td>
<td>RP</td>
<td>GTTGTAAACCTCGCTCTGCTGC</td>
<td></td>
</tr>
<tr>
<td>HS-MIN SAT</td>
<td>FP</td>
<td>GCATGTAAGGACACACCACAC</td>
<td>160</td>
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<tr>
<td></td>
<td>RP</td>
<td>ACTGGAATGTCTGGCAGGAA</td>
<td></td>
</tr>
<tr>
<td>HS-LINE-1</td>
<td>FP</td>
<td>CGAGGTTGAATCGAGAA</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>RP</td>
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<td>HS- IAP1</td>
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<tr>
<td>GAPDH-Mouse</td>
<td>FP</td>
<td>TGCACTCAGCAACTGCCTAG</td>
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<tr>
<td></td>
<td>RP</td>
<td>GGGATGCGGAGGATGATGTTTC</td>
<td></td>
</tr>
<tr>
<td>GAPDH-Human</td>
<td>FP</td>
<td>ACACCCACTCCCTCACCCTT</td>
<td>121</td>
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<tr>
<td></td>
<td>RP</td>
<td>TGACAAGTGCTGCTTCGG</td>
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<tr>
<td>Cullin-1</td>
<td>FP</td>
<td>TTACATCGGAACTGCTCAGG</td>
<td>159</td>
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<td></td>
<td>RP</td>
<td>GGGAGCCCATGTATGGACTT</td>
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<tr>
<td>Cullin-3</td>
<td>FP</td>
<td>ACAGCTCACAATCCAGCATC</td>
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<td></td>
<td>RP</td>
<td>CTTGCAATATGTGCTTCCGT</td>
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<tr>
<td>Skp-1</td>
<td>FP</td>
<td>GGAAGATTTGGGAATGGATG</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td>ATCTTCAGGAGGAGGAGGG</td>
<td></td>
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</tbody>
</table>
Reaction Mix for strand specific cDNA synthesis

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X Reverse transcriptase buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>4 μl</td>
</tr>
<tr>
<td>dNTP's Mix (2.5 mM of each ATP, CTP, TTP, GTP)</td>
<td>4 μl</td>
</tr>
<tr>
<td>Primer 1 (10 mM of Ginir or Giniras-specific)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Primer 2 (10 mM of GAPDH specific)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>RNasin (40 U/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Reverse Transcriptase (15 U/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>RNA</td>
<td>2 μg</td>
</tr>
<tr>
<td>Nuclease Free Water</td>
<td>Upto 20 μl</td>
</tr>
</tbody>
</table>

Reaction was incubated at 42°C for 70 min. Reaction was stopped heating reaction mix for 5 min at 95°C. Strand-specific cDNA was stored at -20°C. Quantitative PCR was done with POWER SYBR green master mix (Invitrogen) on ABI Fast 7500 (Applied Biosystems). The methodology used to make strand specific cDNA is briefly described in Fig 2.2.

![Flowchart showing methodology used for strand specific cDNA synthesis and also showed that cDNA for Ginir and Giniras are complementary to their original sequence.](image-url)
Real time Reaction Mix:

- 2X POWER Sybr green master mix- 5 μl
- Forward Primer- 0.2 μl
- Reverse Primer- 0.2 μl
- cDNA (1:10 diluted) - 1.0 μl
- Nuclease Free Water Upto 10 μl

Cycling conditions were initial 95°C/2 min; 40 cycles of 94°C/30 sec, 60°C/30 sec, 72°C/3 min; and final extension at 72°C/10 min; 4°C/hold. Quantitative experiments were performed in triplicate.

Stable clones for Ginir and Giniras generated in NIH and SK-N-MC Ginir and Giniras, were tested for over-expression of their respective transcripts by performing real-time analyses and by calculating fold change values. For calculating fold change, all results from three technical replicates were normalized to GAPDH and expressed as ΔCt values.

\[ \Delta C_t = C_{(GAPDH)} - C_{(Ginir/Giniras)} \]

Fold change was calculated using ΔΔCt method.

\[ \Delta \Delta C_t = \Delta C_t \text{(Control cells)} - \Delta C_t \text{(Ginir over-expressing cells)} \]

Fold Change = \(2^{\Delta \Delta C_t}\)

Experiment was performed independently using stable clones selected from three independent transfections. Data are presented as mean ± standard error of the mean (SEM). Differences were considered significant with P<0.05.

For performing real-time analyses of mouse embryo, we employed absolute copy number approach. Ginir and GAPDH were amplified by PCR and were gel purified. Amount of DNA in each sample was quantified using nanodrop and from that copy number of DNA was calculated using formula as given below

\[ \text{Number of copies} = \left( \frac{\text{amount of DNA} \times 6.022 \times 10^{23}}{\text{length} \times 1 \times 10^9 \times 650} \right) \]

Where amount of DNA is represented as ng/μl, 6.022x10^{23} is Avogadro’s number, Length is Length of DNA and 650 is molecular weight of each base pair based on their size.

For absolute quantification of Ginir and Giniras copy number was calculated based on their Ct value. Relative expression change was plotted as graph by calculating ratio of Log (Copy number of Ginir/Giniras): Log (Copy number of GAPDH). For performing real time expression analysis of LINE-1, IAP-1, Min SAT, Cullin-1, Cullin-3 and Skp-1 in various cell systems, experiment was done similarly except cDNA synthesis. For detecting of multiple transcripts, we prepared cDNA using oligo dT and random hexamer primers as follow:
5 X Reverse transcriptase buffer 5 µl
25mM MgCl₂ 4 µl
dNTP’s Mix (2.5mM of each ATP, CTP, TTP, GTP) 4 µl
Oligo dT Primer (0.5 µg/µl) 0.5 µl
Random hexamer Primer (0.5 µg/µl) 0.5 µl
RNasin (40 U/µl) 1 µl
Reverse Transcriptase (15 U/µl) 1 µl
RNA 2 µg
Nuclease Free Water Upto 20 µl

Data was represented as a function of fold change as described above from three technical replicates (n=3). Mean± SEM was calculated for the expression change and data with p value> 0.05 was considered significant.

![Standard Plot of Ginir/Giniras](image1)

![Standard Plot of GAPDH](image2)

**Fig. 2.3** Standard curve for Ginir and GAPDH demonstrating a linear relationship between logarithm of copy number and Ct. The standard curve for Ginir/Giniras and GAPDH was linear over six to six orders of magnitude, respectively.

### 2.10. RNA in situ hybridization

**Reagents and Solutions**

#### 4% Para-formalehyde Solution (PFA)

As described in section 2.1.

#### Permeabilization buffer (0.1% Triton X-100 in 1X PBS)

10 µl Triton X-100 (Sigma) was added in 10 ml of 1X PBS. Solution was stored at room temperature for future use.
20X SSC (Saline- sodium citrate) solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>75.3 g</td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td>88.2 g</td>
</tr>
</tbody>
</table>

Reagents were dissolved in 80 ml deionized water and pH was adjusted to 7.0 using 1N HCl solution. Volume was adjusted using deionized water to 1 L and autoclaved for sterilization. The solution was aliquoted and kept in 4ºC for further use.

Blocking buffer (5% BSA in 4X SSC)

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA powder (Sigma)</td>
<td>5 g</td>
</tr>
<tr>
<td>20X SSC</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

Reagents were mixed and volume was made up to 10 ml with deionised water. Solution was always prepared fresh.

20% Dextran Sulphate

2 g Dextran sulphate (Sigma) was dissolved in 7 ml of deionised water and volume was made up to 10 ml. 1 ml aliquots prepared and stored at -20ºC.

Pre-hybridization buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% dextran sulphate-</td>
<td>5 ml</td>
</tr>
<tr>
<td>20X SSC-</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10 mg/ml Salmon sperm DNA (Sigma)</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Volume was made up to 10 ml using deionised water and 1 ml aliquots were stored in -20ºC for future use.

Locked Nucleic Acid (LNA) probes for Ginir and Giniras

Using Exiqon services, customized LNA probes for Ginir and Giniras were synthesized which contained fluorescent tags FAM and TexRed, respectively.

LNA probes synthesis

Table 2.1.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Fluorescent Tag</th>
<th>Deionized water (Final conc. 25 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginir</td>
<td>GGTGGCCTTTCCTTCAGT</td>
<td>56-FAM</td>
<td>200 µl</td>
</tr>
<tr>
<td>CTCT</td>
<td>CATCCAGTAGATAGTCAC AGCC</td>
<td>TexRed</td>
<td>143 μl</td>
</tr>
<tr>
<td>------</td>
<td>--------------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
</tbody>
</table>

**Hybridization buffer**

LNA-probe for Ginir 1 μl  
LNA-probe for Giniras 1 μl  
Reagents were mixed thoroughly with 1 ml of pre-hybridization buffer and used immediately for hybridization.

**Wash buffer-I (4X SSC in 0.1% Triton X-100)**

| 20X SSC | 2 ml  
Triton X-100 | 10 μl  
Reagents were mixed and volume of final solution was made upto 10 ml. Solution was always prepared fresh.

**Wash buffer-II (2X SSC)**

| 20X SSC | 1 ml  
Reagent was mixed with 9 ml of deionised water. Solution was always prepared fresh.

**Wash buffer-III (1XSSC)**

0.5 ml 20X SSC was added to 9.5 ml of deionised water. Solution was always prepared fresh.

**100μM Sodium Citrate (pH 6.0)**

**Stock Solution**

2.94 g tri-sodium citrate powder (Qualigens) was dissolved in 100 ml of deionized water and pH was adjusted to 6.0 using 1 N HCl (Fisher scientific). The volume was made upto 100 ml using deionized water and solution was stored at 4°C.

**Working Solution**

Stock solution was diluted 1:10 and use immediately.

**10mM Sodium Actetate solution pH 5.2**

140 mg sodium acetate trihydrate (Fisher scientific) was dissolved in 80 ml of deionized water and pH was adjusted to 5.2 using glacial acetic acid. Volume
was made up to 100 ml with deionized water. Solution was autoclaved and kept at room temperature for further use.

1M Tris-HCl pH7.4
12.1 g Tris base powder was dissolved in 40 ml of deionised water; pH was adjusted to 7.4 with conc. HCl. The volume was made up to 100 ml; solution was stored at 4°C.

RNase A Solution (10 mg/ml)
RNase A powder (Sigma Aldrich) 0.5 g
10mM sodium acetate buffer (pH 5.2) 35ml
Reagents were mixed and heated at 100°C for 15 min in 10mM Sodium Acetate solution pH5.2. Solution was allowed to cool at room temperature and pH was adjusted to 7.4 using ~ 10 ml of 1M Tris-HCl buffer, pH 7.4. RNase A solution was aliquoted to 1.5 ml tubes and kept at -20°C until use.

Methodology
Cells grown on coverslips in a 24 well plate were washed with 1X PBS twice and fixed in 4% para-formaldehyde for 10 min. Cells on coverslips were then washed with 1X PBS and blocking buffer was added to it for 30 min at room temperature. 200 µl of pre-hybridization buffer was added for 30 min at 42°C to equilibrate the samples. 100 µl of hybridization buffer containing Ginir and Giniras probes for detecting sense and antisense transcripts were added to each coverslip for 3 h at 42°C. Coverslips were washed with Wash buffer-I three times for 5 min at 45°C. Further, one wash was given with Wash buffer-II, Wash buffer–III and 1X PBS each for 5 min at 45°C. DAPI was then added to coverslips at room temperature for 5 min followed by three washes with 1X PBS at intervals of 5 min each. Cells were mounted with mounting medium (Sigma) containing 10% DABCO (1, 4-diazabicyclo [2.2.2] Octane (Sigma). used as anti-fade reagent (Sigma). For negative control, RNase A treatment was given to cells for 30 min at 37°C prior to addition of pre-hybridization buffer. Samples were acquired using Olympus FV10i Fluoview confocal microscope (Olympus, Tokyo, Japan) at 60X. Samples were acquired using 405 nm, 488 nm and 594 nm lasers for DAPI, Ginir probe and Giniras probe. DAPI was used as nuclear stain.

For in situ hybridization of Ginir and Giniras, embryos from 12.5 dpc to 17.5 dpc were isolated and fixed in 4% para-formaldehyde overnight at 4°C. 10 µm paraffin sections of mouse embryo were given for sectioning to KEM hospital,
Pune, India. To perform hybridizations, sections were de-waxed by putting slides stepwise in Xylene for 10 min and in gradient of ethanol (100%, 95%, 80% and 70%) for 5 min each. Sections were then washed with 1X PBS and were incubated in fumes of 10µM of sodium citrate (pH 6.0) for 10 min for antigen retrieval which were then again washed with 1X PBS. After that same procedure of pre-hybridization, hybridization and washing was followed as discussed above. Here also, RNAse A treatment prior to hybridizations was used as negative control. Samples were acquired using Olympus FV10i Fluoview confocal microscope at 4X and 10X (Olympus,).

For whole mount in situ hybridizations for Ginir and Giniras, embryos from 8.5 dpc to 11.5 dpc were isolated and fixed in 4% para-formaldehyde for overnight at 4ºC. 12-18 h later, 0.05% pepsin treatment in 0.01 N HCl was given for 5-15 min, depending on size of embryo, for better penetration of the probe in embryos. After pepsin treatment, embryos were thoroughly washed with 1X PBS and blocked using blocking buffer for 30 min at room temperature in a 2 ml tube. Blocking buffer was removed using 200 µl pipette very carefully and 200 µl of pre-hybridization buffer was added for 30 min at 42ºC to equilibrate the samples. 100 µl of hybridization buffer for detecting sense and antisense transcript was added to each tube containing embryos for 8 h at 42ºC. Embryos were washed with wash buffer-I three times and each wash was for 15 min at 45ºC. One wash was given with 2X SSC, 1X SSC and 1X PBS each for 15 minutes at 45ºC. DAPI was then added to embryos at room temperature for 5 minutes and then three washes with 1X PBS were given for 5 minutes each. Embryos were kept in 35 mm plastic plates and immediately preceded for imaging using IX81 inverted microscope (Olympus) at 10X. For negative control, before incubating the embryos with hybridization buffer, pre-hybridization buffer was supplemented with RNAse A. Samples were acquired using fluorescent microscope at 10X at 60X. For long times storage samples were dehydrated with gradient of methanol by keeping embryos after fixation in 25%, 50% and 75% methanol (Merck) for 10 minutes each. Finally embryos were stored in 100% methanol. These embryos can be stored in -20ºC for at least a year. For performing hybridizations embryos were rehydrated through a gradient of methanol containing 75%, 50% and 25% of methanol and finally kept in 1X PBS. After rehydration, same procedure was followed.
2.11. Immuno- fluorescence staining

Reagents and Solutions

1X PBS
Solution was prepared as described in section 2.1.

4% Para-formaldehyde Solution (PFA)
Solution was prepared as described in section 2.1.

Permeablization buffer (0.1% Triton X-100 in 1X PBS)
Solution was prepared as described in section 2.10.

Blocking buffer (5% BSA in 1XPBS)
BSA powder (Sigma) 0.5 g
5X PBS 2 ml
Reagents were dissolved in 5 ml water and volume was made up to 10 ml with deionised water. Solution was stored at -20ºC.

Table 2.2:
List of antibodies used for immuno-fluorescence along with details of their incubation time, temperature and also secondary antibody used against them.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Company</th>
<th>Dilution; Time; Temp.</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct-4</td>
<td>SantaCruz Biotech. Inc(SC, USA)</td>
<td>1:50; O/N; 4ºC</td>
<td>Anti-mouse AlexaFlour 488 (Invitrogen)</td>
</tr>
<tr>
<td>Sox-2</td>
<td>SantaCruz Biotech. Inc</td>
<td>1:50; overnight; 4ºC</td>
<td>Anti-Rabbit AlexaFlour 594 (Invitrogen)</td>
</tr>
<tr>
<td>Nanog</td>
<td>Millipore, MA, USA</td>
<td>1:200; overnight; 4ºC</td>
<td>Anti-Mouse AlexaFlour 488</td>
</tr>
<tr>
<td>SSea-4</td>
<td>Millipore</td>
<td>1:100; overnight; 4ºC</td>
<td>Anti-Mouse AlexaFlour 594</td>
</tr>
<tr>
<td>Protein</td>
<td>Antibody Provider</td>
<td>Dilution</td>
<td>Incubation Details</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------</td>
<td>------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>Tra-181</td>
<td>Millipore</td>
<td>1:100;</td>
<td>overnight; 4ºC</td>
</tr>
<tr>
<td>ABCG-2</td>
<td>Abcam, MA, USA</td>
<td>1:100;</td>
<td>overnight; 4ºC</td>
</tr>
<tr>
<td>CD133</td>
<td>Abcam</td>
<td>1:100;</td>
<td>overnight; 4ºC</td>
</tr>
<tr>
<td>pATM-Ser</td>
<td>Cell Signalling, MA, USA</td>
<td>1:100;</td>
<td>2h; room temp</td>
</tr>
<tr>
<td>γnti-R</td>
<td>Cell Signalling</td>
<td>1:100;</td>
<td>2h; room temp</td>
</tr>
<tr>
<td>BMI-1</td>
<td>Upstate</td>
<td>1:100;</td>
<td>2h; room temp</td>
</tr>
<tr>
<td>Mitf</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:100;</td>
<td>1h; room temp</td>
</tr>
<tr>
<td>Actinin</td>
<td>Abcam</td>
<td>1:100;</td>
<td>1h; room temp</td>
</tr>
<tr>
<td>MyoD1</td>
<td>SantaCruz Biotech. Inc</td>
<td>1:100;</td>
<td>1h; room temp</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>SantaCruz Biotech. Inc</td>
<td>1:100</td>
<td>overnight; 4ºC</td>
</tr>
<tr>
<td>Pdx-1</td>
<td>Abcam</td>
<td>1:200;</td>
<td>1h; room temp</td>
</tr>
<tr>
<td>AFP</td>
<td>Santacruz Biotech. Inc</td>
<td>1:200;</td>
<td>3h; room temp</td>
</tr>
<tr>
<td>Sox-17</td>
<td>Santacruz Biotech. Inc</td>
<td>1:100;</td>
<td>3h; room temp</td>
</tr>
<tr>
<td>GFAP</td>
<td>Abcam</td>
<td>1:100;</td>
<td>1h; room temp</td>
</tr>
<tr>
<td>Tuj-1</td>
<td>Millipore</td>
<td>1:100;</td>
<td>1h; room temp</td>
</tr>
</tbody>
</table>
Methodology

Cells were grown on cover-slips in 24 well plates for 24 h. Cells were washed with ice cold 1X PBS and fixed in 4% Para-formaldehyde for 10 min at room temperature. After two rinses in 1X PBS, cells were permeabilized with permeabilization buffer for 10 min. Non specific antigens were blocked using blocking buffer in 1X PBS for 30 min at room temperature. This was followed by incubation with a panel of primary antibodies as listed in table 2.2. The cells were then washed with 1X PBS three times and then incubated with appropriate Alexa Fluor-conjugated secondary antibody (Molecular Probes, Invitrogen) diluted in 1% BSA in 1x PBS (1:100 dilutions) for 1 h at room temperature in dark. Cells were washed three times in PBS in the dark and then incubated with DAPI (4', 6-diamidino-2-phenylindole) for 5 mins. Cells were mounted with mounting medium containing DABCO (1, 4-diazabiclo [2.2.2] Octane (Sigma,)). Confocal images were acquired using Zeiss LSM510 system microscope. Embryoid-like bodies were stained by directly spinning them onto glass slides coated with poly-L-Lysine (Sigma) using Cytospin at 1000 X g for 5 minutes.

2.12. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

Reagents and Solutions for SDS-PAGE

29% Acrylamide + 1% Bis-acrylamide
Acrylamide (Sigma Aldrich) 29.0 g
N, N-Methylene bisacrylamide (Sigma Aldrich) 1.0 g
The volume was made to 100 ml with deionised water. The solution was filtered and stored in dark at 4°C.

4X Tris-HCl, pH 6.8 (0.5 M Tris-HCl)
6.05 g Tris base powder was dissolved in 40 ml of deionised water; pH was adjusted to 6.8 with 1 N HCl. The volume was made upto 100 ml. The solution was stored at 4°C.

4X Tris-HCl, pH 8.8 (1.5 M Tris-HCl)
18.17 g Tris base powder was dissolved in 40 ml of deionised water; pH was adjusted to 8.8 with 1 N HCl. The volume was made upto 100 ml. The solution
was stored at 4°C.

10% Sodium Dodecyl Sulphate (SDS) Solution
10g SDS powder was dissolved in 80 ml of deionised water and the volume was made upto 100 ml with deionised water and stored at room temperature.

10% (w/v) Ammonium Persulphate
0.1 g Ammonium persulphate (USB) powder was dissolved in 1 ml of deionised water. The solution was prepared fresh every time.

5X Sample Buffer (SDS-PAGE)
4X Tris HCl, pH 6.8 12.5 ml
Glycerol (Fisher Scientific) 8.3 ml
SDS (USB) 3.0 g
2-Mercaptoethanol (Sigma Aldrich) 1.25 ml
Bromophenol blue (Sigma Aldrich) 0.125 g
These reagents were dissolved in deionised water. Volume was made to 25 ml with deionised water.

10 X Electrophoresis Buffer
Glycine (USB) 144 g
Tris base 30.3 g
SDS 10 g
These reagents were dissolved in deionised water and the volume was made to 1000 ml with deionised water.

SDS-PAGE Gel Composition

<table>
<thead>
<tr>
<th>Stacking gel (2.5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>30% Acrylamide + 0.8% Bis-acrylamide</strong></td>
</tr>
<tr>
<td><strong>4X Tris-HCl (0.5 M Tris-HCl, pH 6.8)</strong></td>
</tr>
<tr>
<td><strong>Deionised water</strong></td>
</tr>
<tr>
<td><strong>10% SDS</strong></td>
</tr>
<tr>
<td><strong>10% Ammonium per sulphate</strong></td>
</tr>
</tbody>
</table>
## Reagents and Solutions for Western Blotting

### 1X Transfer Buffer
- **Tris base**: 3.03 g
- **Glycine**: 14.4 g
- **Methanol (Fisher Scientific)**: 200 ml
- Deionised water was added to make volume 1000 ml

### 1X Phosphate Buffered Saline (PBS)
Solution was prepared as described in section 2.1.

### 1X Tris Buffered Saline (TBS)
- **NaCl**: 8.0 g
- **KCl**: 0.2 g
- **Tris base**: 3.0 g

These chemicals were dissolved in 800 ml deionised water and pH was adjusted to 7.4 with 1 N HCl. The volume was made up to 1000 ml.

---

<table>
<thead>
<tr>
<th>Resolving gel (9.0 ml)</th>
<th>7.5%</th>
<th>10%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>29% Acrylamide +</strong></td>
<td>2.25 ml</td>
<td>3.0 ml</td>
<td>4.08 ml</td>
</tr>
<tr>
<td><strong>1.0 % Bis-acrylamide</strong></td>
<td>ml</td>
<td>ml</td>
<td></td>
</tr>
<tr>
<td><strong>4X Tris-HCl (1.5 M Tris-HCl, pH 8.8)</strong></td>
<td>2.25 ml</td>
<td>2.2 ml</td>
<td>2.25 ml</td>
</tr>
<tr>
<td><strong>Deionized water</strong></td>
<td>4.50 ml</td>
<td>3.7 ml</td>
<td>2.97 ml</td>
</tr>
<tr>
<td><strong>10% SDS</strong></td>
<td>90 µl</td>
<td>90 µl</td>
<td>90 µl</td>
</tr>
<tr>
<td><strong>10% Ammonium per sulphate</strong></td>
<td>60 µl</td>
<td>60 µl</td>
<td>60 µl</td>
</tr>
<tr>
<td><strong>TEMED</strong></td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
**Blocking Solution**
5% non-fat dry milk was dissolved in TBS-T.

**Stripping Buffer**
62.5 mM Tris-HCl, pH 6.7
2% SDS
100 mM 2-mercaptoethanol
Finally the volume was made to 100% with deionised water.

**Methodology**
Cells were washed twice with ice cold 1X PBS. Cells were harvested and resuspended in appropriate amount of ice-cold lysis buffer (Pierce, IL, USA) (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40(NP 40), 0.5% sodium deoxycholate, and 5 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min to allow complete lysis. The cell lysates were cleared by centrifugation at 12000xg for 15 min at 4°C and the concentration of protein in clear cell lysates was measured by Bio-Rad protein assay (Bio-Rad, CA, USA). Equal concentrations of total proteins were mixed with 8 µl of 5X sample buffer and deionised water was added to make final volume up to 40 µl. The samples were boiled at 95°C for 5 min. The proteins were resolved by SDS-PAGE using Bio-Rad mini-gel electrophoresis unit. For western blot analysis, the proteins were electro-transferred to PVDF membrane (Millipore) using a mini trans-blot electrophoretic transfer apparatus. The membranes were blocked with 5.0% non fat dried milk in TBS-T for 1 h at room temperature. The membranes were rinsed with TBS-T and incubated with primary antibodies as enlisted in Table 2.3 at required dilution in TBS-T for 3 h at room temperature or overnight at 4°C. Blots were then washed thrice with TBS-T followed by incubation with HRP conjugated anti-rabbit/anti-mouse/anti-goat IgG antibody for 1 h. The blots were again washed thrice with TBS-T and detected by Pierce Femto Western Blot detection kit (Pierce). Peroxide solution and enhancer solution were mixed 1:1. The mix was spread onto blot uniformly and kept without disturbing for 5 min. After 5 min, excess of solution was discarded and blots were kept between two transparencies. Signal was captured onto X-ray film at different exposures. For re-probing, the membranes were incubated in stripping buffer at 50°C for 30 min.
and then washed thrice with TBS-T at room temperature for 10 min. The membranes were then blocked and reprobed with different primary antibodies.

Table 2.3 List of primary and secondary antibodies used for Western Blotting along with details.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution; Time; Temp</th>
<th>Secondary Antibody</th>
<th>Dilution; Time; Temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>γH2AX</td>
<td>Chemicon</td>
<td>1:1000; overnight; 4°C</td>
<td>Anti-Mouse (Pierce)</td>
<td>1:2000; 90 min; RT</td>
</tr>
<tr>
<td>pATM</td>
<td>Cell Signalling</td>
<td>1:2000; 2h; RT</td>
<td>Anti-Rabbit (Pierce)</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>pATR</td>
<td>Cell Signalling</td>
<td>1:2000; 2h; RT</td>
<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>pChk-1</td>
<td>Cell Signalling</td>
<td>1:2000; 2h; RT</td>
<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>pChk-2</td>
<td>Cell Signalling</td>
<td>1:2000; 2h; RT</td>
<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>p53</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:3000; 1h; RT</td>
<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>pp53</td>
<td>Cell Signalling</td>
<td>1:2000; 2h; RT</td>
<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>p16</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:2000; 2h; RT</td>
<td>Anti-Mouse</td>
<td>1:2000; 90 min; RT</td>
</tr>
<tr>
<td>p19</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:2000; 2h; RT</td>
<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>Gli-1</td>
<td>Cell Signalling</td>
<td>1:2000; 3h; RT</td>
<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>Twist</td>
<td>Abcam</td>
<td>1:1500; 3h; RT</td>
<td>Anti-Mouse</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>BMI-1</td>
<td>Millipore</td>
<td>1:2000; 2h; RT</td>
<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
</tr>
<tr>
<td>Pp38</td>
<td>Cell Signalling</td>
<td>1:1000;</td>
<td>Anti-</td>
<td>1:6000;</td>
</tr>
<tr>
<td>Protein</td>
<td>Source</td>
<td>Dilution</td>
<td>Incubation</td>
<td>Antibody</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------</td>
<td>----------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Cell Signalling</td>
<td></td>
<td>overnight;</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pAkt 473</td>
<td>Cell Signalling</td>
<td>1:1000;</td>
<td>overnight;</td>
<td>Anti-Rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4ºC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akt</td>
<td>Cell Signalling</td>
<td>1:1000; 3h;</td>
<td>RT</td>
<td>Anti-Rabbit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Cell Signalling</td>
<td>1:2000; 2h;</td>
<td>RT</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:2000; 2h;</td>
<td>RT</td>
<td>Anti-Mouse</td>
</tr>
<tr>
<td>Cdk-4</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:2000; 2h;</td>
<td>RT</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Cdk-2</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:2000; 2h;</td>
<td>RT</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Cullin-1</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:1000; 3h;</td>
<td>RT</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Cullin-3</td>
<td>Sanatcruz Biotech. Inc</td>
<td>1:1000; 3h;</td>
<td>RT</td>
<td>Rabbit</td>
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<tr>
<td>FBXO 31</td>
<td>Sigma Aldrich</td>
<td>1:1000; 3h;</td>
<td>RT</td>
<td>Rabbit</td>
</tr>
<tr>
<td>FBXW 7</td>
<td>Abcam</td>
<td>1:2000; 3h;</td>
<td>RT</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Skp-1</td>
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<td>RT</td>
<td>Rabbit</td>
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<td>PCNA</td>
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<tr>
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<td>Oct-4</td>
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<td>Anti-Mouse</td>
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<td>Anti-Rabbit</td>
<td>1:6000; 1h; RT</td>
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<td>Gene</td>
<td>Vendor</td>
<td>Dilution</td>
<td>Incubation Temp</td>
<td>Incubation Time</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------</td>
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<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
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<td>Millipore</td>
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<td>overnight;</td>
<td>4ºC</td>
</tr>
<tr>
<td></td>
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<td>overnight;</td>
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<tr>
<td>Nanog</td>
<td>Millipore</td>
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<td>overnight;</td>
<td>4ºC</td>
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<tr>
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<td>overnight;</td>
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<tr>
<td>β_Actin</td>
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<tr>
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<tr>
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<td>O/N;</td>
<td>4ºC</td>
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<td>O/N;</td>
<td></td>
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<tr>
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<td>4ºC</td>
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<td>PECA</td>
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<td>4ºC</td>
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<td>Sox-17</td>
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<td>O/N;</td>
<td>4ºC</td>
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<tr>
<td>Pdx-1</td>
<td>Abcam</td>
<td>1:3000;</td>
<td>O/N;</td>
<td>4ºC</td>
</tr>
</tbody>
</table>

2.13. FACS analysis

Reagents and Solutions

1X PBS
Solution was prepared as described in section 2.1.

4% Para-formalehyde Solution (PFA)
Solution was prepared as described in section 2.1.
Permeabilization buffer (0.1% Triton X-100 in 1X PBS)
Solution was prepared as described in section 2.11.

Blocking buffer (5% BSA in 1XPBS)
Solution was prepared as described in section 2.11.

Methodology
SK-MC-EV, SK-MC-Ginir and SK-MC-EV-Giniras cells were cultured in a 60 mm dish and after 70-80% confluency, cells were trypsinized using TPVG followed by washing with 1X PBS containing 0.2% FBS. Cells were then blocked, fixed using 4% Paraformaldehyde solution for 10 min at room temperature. For BMI-1 and Twist staining, cells were permeabilized with 0.1% Triton X-100 and were blocked with blocking buffer for 30 min. Primary antibody was added at a concentration of 1:100 for 90 minutes at room temperature and secondary antibodies were added at a concentration of 1:200 for 45 minutes at room temperature (RT). After Primary and secondary antibody incubations cells were washed twice with 1X PBS. Cells were acquired using BD Biosciences FACSCanto II flow cytometer at 488 and 594 nm lasers (BD Biosciences) and data was analysed using BD FACSDiva™ Software v (BD Biosciences).

Table 2.4 List of primary and secondary antibodies used for FACS analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Dilution; Time; Temp</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI-1</td>
<td>Millipore</td>
<td>1:100; 3h; RT</td>
<td>Anti-mouse AlexaFlour 488 (Invitrogen)</td>
</tr>
<tr>
<td>Twist</td>
<td>Abcam</td>
<td>1:100; 3h; RT</td>
<td>Anti-Rabbit AlexaFlour 594 (Invitrogen)</td>
</tr>
</tbody>
</table>

2.14. Chromatin Immuno-precipitation (CHIP) assay
Reagents and Solutions

Chromatin immunoprecipitation (CHIP) assay kit (Upstate biotechnology, NY, USA) contains following components which were stored at 4°C.

Salmon Sperm DNA/Protein A Agarose,
Three vials, each containing 500 ml packed beads with 200 mg sonicated salmon sperm DNA, 500 mg BSA, ~1.5 mg recombinant protein A. Provided as a 50% gel slurry in TE buffer containing 0.05% sodium azide; final volume of 1 ml.

**Dilution Buffer**
Two vials, each containing 25 ml of 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl.

**Low Salt Immune Complex Wash Buffer** One vial containing 25 ml of 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl.

**High Salt Immune Complex Wash Buffer**
One vial containing 25ml of 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl.

**LiCl Immune Complex Wash Buffer**
One vial containing 25 ml of 0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1.

**1X TE Buffer**
Two vials, each containing 25 ml of 10mM Tris-HCl, 1mM EDTA, pH 8.0.

**5M NaCl**
One vial containing 500 ml of 5M NaCl.

**0.5M EDTA**
One vial containing 250 ml of 0.5M EDTA, pH 8.0.

**1M Tris-HCl, pH 6.5**
One vial containing 500 ml of 1M Tris-HCl, pH 6.5.

**SDS Lysis Buffer**
One vial containing 10 ml of 1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1.

**1% Formaldehyde solution**
500 μl formaldehyde (40% stock) (Fisher-scientific) was added to 19.5 ml of culture medium. Solution was always prepared fresh before use.

**1X PBS**
Solution was prepared as described in section 2.1.

**Elution buffer (1%SDS, 0.1M NaHCO3)**
- SDS: 0.1 g
- 1M Sodium bicarbonate (Sigma Aldrich): 1.0 ml

Reagents were dissolved in 8 ml deionized water and final volume was made up to 10 ml using deionized water.
10 μg / μl Proteinase K

100 μg of Proteinase K (Sigma Aldrich) was dissolved in 10 ml deionized water. Solution was aliquoted and stored at -20°C.

**Methodology**

To check whether down-regulation of p16 and p19 in SK-N-MC Ginir cells was due to epigenetic modifications of chromatin, we performed CHIP assay for Ub-H2A. 1 x 10⁶ SK-N-MC EV and SK-N-MC Ginir cells were seeded in 100 mm dish. Cross linking of all proteins to DNA was done by adding formaldehyde directly to culture medium to a final concentration of 1% and incubated for 20 min at 37°C. Medium was removed and cells were washed with ice cold 1X PBS containing protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1mg/ml aprotinin and 1mg/ml pepstatin A) (Sigma). Cells were scraped, collected and pelleted for 4 minutes at 700xg at 4°C. Supernatant was decanted and 200 μl SDS Lysis Buffer was added to the pellet and kept for 10 min on ice. Lysates were sonicated to obtain DNA between 200 and 1000 bp by putting the power of sonicator at 4 (40% of Maximum power) and by giving 10 pulses of 10 seconds thrice. Samples were cooled on ice between pulses for 30 seconds. To remove debris from the sample, centrifugation was performed for 10 min at 21,000xg at 4°C. Supernatant was diluted 10 fold in CHIP Dilution Buffer and protease inhibitors were added to it as mentioned earlier. 200 μl of this chromatin solution (1%) was kept separately as input to quantitate the amount of DNA present in different samples before immunoprecipitation. To the rest of the sample, preclearing for non-specific proteins was done using 20 μl of salmon sperm DNA/Protein A Agarose slurry (Invitrogen). Beads were separated from the sample by spinning the tubes at 10,000xg for 1 min and supernatants were collected in new tubes. 2 μg of H2A-ub (Abcam) antibody was added to 1 ml of supernatant and incubated overnight at 4°C with rotation. To the other 1 ml of chromatin solution non-specific antibody was added. The complexes formed were captured by adding Salmon Sperm DNA/Protein A Agarose Slurry for one hour at 4°C with rotation. Beads were again collected by spinning down at 10,000xg for 1 min and washed for 5 min each with Low salt, then High salt and then LiCl buffer. Two more washes were given with 1X TE and elution was done by adding 250 ml elution buffer to pelleted beads. Samples were briefly vortexed to mix and incubated at room temperature for 15 min with rotation. Beads were spun down and supernatant fraction was transferred to fresh tube. Elution was repeated once more. Both elutes were combined and 20 μl of 5M NaCl was...
added. Tubes were kept at 65ºC for 4 h for reverse cross linking. To the solution 1 µl of 0.5M EDTA, 2 µl of 1M Tris-HCl, pH 6.5 and 2 µl of 10 μg/μl Proteinase K was added and incubated for one hour at 45ºC. DNA was recovered by phenol/chloroform extraction and ethanol precipitation by addition of 20 μg glycogen. After washing pellets with 70% ethanol, they were resuspended in nuclease free water. Bands for p16 and p19 were detected by performing PCR using primers listed in table.

2.15. RNA pull down assay

**Reagents and Solutions**

**Biotin-CTP**
CTP tagged with biotin was commercially procured from Invitrogen.

**MegaScript in vitro transcription (Invitrogen)**
This kit was procured from Invitrogen and contained following components

- Enzyme Mix (T7) 50 µl
- 10X Reaction Buffer 50 µl
- ATP Solution 50 µl
- CTP Solution 50 µl
- GTP Solution 50 µl
- UTP Solution 50 µl
- Nuclease-free Water 175 µl
- TURBO DNase (2 U/µL) 100 µl
- Ammonium Acetate Stop Solution 1.0 ml
- Lithium Chloride Precipitation Solution 1.0 ml
- Gel Loading Buffer II 1.4 ml

**1X Phosphate Buffered Saline (PBS)**
Solution was prepared as described in section 2.1.

**Pierce IP lysis buffer** (Pierce)
This buffer was purchased commercially from Pierce and was used after adding protease inhibitor cocktail (Sigma)

**µMACS Streptavidin Kit** (Miltenyi Biotec)
Components of this kit are as follow:

- **µMACS™ Streptavidin MicroBeads**: 2.0 ml
- **Equilibration Buffer for protein**: 4.0 ml
- **Equilibration Buffer for nucleic acid**: 4.0 ml
- **µColumns**: 20

1M Tris HCl pH 7.0
12.1 g Tris base powder was dissolved in 50 ml of deionized water and pH was adjusted using conc. HCl to 7.0. Solution was made up to 100 ml and autoclaved for sterilization and kept at 4°C for future use.

1M KCl
7.46 g KCl powder was dissolved in 95 ml H2O and volume was brought up to 100 ml using deionized water. Sterilization was done by autoclaving and solution was kept at 4°C for future use.

1M MgCl₂
2.03 g MgCl₂ powder was dissolved in 95 ml H2O and volume was brought up to 100 ml using deionized water. Sterilization was done by autoclaving and solution was kept at 4°C for future use.

2.5 M NaCl
29.32 g NaCl powder was dissolved in 100 ml of deionized water and sterilized by autoclaving. Solution was kept at room temperature for future use.

RNA binding buffer (10 mM Tris pH 7, 0.1 M KCl, 10 mM MgCl₂)
- 1M Tris HCl pH 7.0: 100 µl
- 1M KCl: 1 ml
- 1M MgCl₂: 100 µl
Reagents were dissolved in 10 ml of deionized water. Solution was always prepared fresh.

RNA-protein elution buffer (10 mM Tris pH 7, 0.1 M KCl, 10 mM MgCl₂, 1M NaCl)
- 1M Tris HCl pH 7.0: 10 µl
- 1M KCl: 100 µl
- 1M MgCl₂: 10 µl
2.5M NaCl 
Volume was made up to 1 ml by adding deionized water. Solution was always prepared fresh.

10X TBE Buffer
Tris base 216 g
Boric acid 110 g
EDTA 6.6 g
Powders were dissolved in 1.5 L of deionized water, by stirring. pH was adjusted using 5N NaOH. Volume was made up to 2 L with deionized water. Buffer was autoclaved and stored at room temperature for future use.

Working solution (0.5X TBE)
10X TBE were diluted 1:20 times with deionized water and kept at room temperature for future use.

Ethidium Bromide (EtBr) solution
10 mg EtBr powder was dissolved in 10 ml of deionized water and aliquoted in 1.5 ml tubes which were stored in dark at room temperature. EtBr is highly carcinogenic so it is advised to take necessary precautions to avoid any skin contact.

1.5% agarose gel
0.75 g Agarose powder was dissolved in 0.5X TBE and heated in microwave oven for 2 min. Mix was allowed to cool and EtBr was added to it. Gel was poured into the casting module and allowed to solidify.

25 mM Ammonium Bicarbonate solution
30 mg ammonium bicarbonate (NH₄HCO₃) was dissolved in 3 ml deionized water. Solution was always prepared fresh.

50 mM Ammonium Bicarbonate solution
60 mg ammonium bicarbonate (NH₄HCO₃) (Sigma) was dissolved 3 ml deionized water. Solution was always prepared fresh.
Dehydration solution (2:1 solution of Acetonitrile: 50mM Ammonium Bicarbonate)

Acetonitrile          6 ml
50 mM Ammonium Bicarbonate solution        3 ml
Regents were mixed in a 15 ml tube and were used as dehydration solution.
Solution was always prepared fresh just before use.

100 mM Ammonium Bicarbonate solution
120 mg ammonium bicarbonate \((\text{NH}_4\text{HCO}_3)\) was dissolved 3 ml Milli-Q water.
Solution was always prepared fresh.

10 mM DTT solution in 100mM ammonium bicarbonate

DTT (Sigma)       1.5 mg
100 mM ammonium bicarbonate        1 ml
Solution was always prepared fresh.

10M iodoacetamide (IAA) solution in 100mM ammonium bicarbonate

IAA (Pierce)       10 mg
100 mM ammonium bicarbonate        1 ml
Solution was always prepared fresh.

0.1% of Tri-Floro acetic acid (TFA) solution
10 \(\mu\)l of TFA (Sigma) was added to 990 \(\mu\)l of deionized water. Solution was always prepared fresh.

Wetting solution:
100% HPLC grade Acetonitrile (ACN) (Pierce) was used as wetting solution

Washing solution:
0.1% TFA solution was used as washing solution. Solution was always prepared fresh.

Elution solution

ACN solution         500 \(\mu\)l
TFA         10 \(\mu\)l
Reagents were dissolved in 490 \(\mu\)l of deionized water. Solution was always prepared fresh.
**Coomassie Blue Staining Solution**

Coomassie blue  0.25%
Acetic acid  10 ml
Methanol  50 ml

Volume was made up with deionised water to 100 ml.

**Destaining Solution**

Acetic acid  10 ml
Methanol  20 ml

Volume was made up with deionised water to 100 ml. Solution was prepared and kept at room temperature.

**In-Gel Tryptic Digestion Kit (Pierce)**

**Kit Contents:**

- Pierce Trypsin Protease MS Grade  20 µg
- Trypsin Storage Solution  40 µl
- Acetonitrile  3 × 24 ml
- Ammonium Bicarbonate  300 mg
- TCEP (Tris[2-carboxyethyl]phosphine)  500 µl
- Iodoacetamide (IAA)  500 mg

**Stock Trypsin Solution**

Lyophilized Pierce Trypsin Protease (MS Grade) was diluted in 20 µl of the supplied Trypsin Storage Solution. To minimize freeze-thaw cycles and to increase storage stability, Stock solution of Trypsin was aliquoted into four separate tubes of 5µL each. Aliquots were stored at -20°C in a nonfrost-free freezer.

**Working Trypsin Solution**

5 µl of stock Trypsin solution was diluted with 45 µl of deionized water to obtain a final concentration of 0.1 µg/µl. This solution was kept at -20°C for not more than 2 months.

**Activated Trypsin Solution**

Working Trypsin solution  10 µl
25 mM Ammonium bicarbonate solution  90 µl.
Both the solutions were mixed properly and always prepared fresh. Final concentration of activated trypsin solution was 10 ng/µl. For each reaction 100 ng of activate trypsin was used.

**Methodology**

**Biotinylation of RNA**
Using pTarget Ginir plasmid, Ginir was PCR amplified using T7 as forward primer. PCR product is purified using PCR purification kit (Qiagen) and 2 µl of product was checked by running onto a 1.5% agarose gel.

**Reaction mix:**

<table>
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<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer (Qiagen)</td>
<td>10 µl</td>
</tr>
<tr>
<td>dNTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>Forward Primer (T7)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>Template</td>
<td>200 ng</td>
</tr>
<tr>
<td>Taq DNA polymerase (Qiagen)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Bio-CTP solution(1:10 diluted)</td>
<td>1.97 µl</td>
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</tbody>
</table>

Cycling conditions were initial denaturation 95°C/5min; 30 cycles of 94°C/30sec, 55°C/30sec, 72°C/45 sec; and final extension at 72°C/10min;4°C/hold. PCR product was used as template for *invitro* RNA transcription of biotinylated Ginir RNA.

**Reverse transcription reaction Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X Reaction Buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>ATP solution</td>
<td>2 µl</td>
</tr>
<tr>
<td>GTP solution</td>
<td>2 µl</td>
</tr>
<tr>
<td>UTP solution</td>
<td>2 µl</td>
</tr>
<tr>
<td>CTP solution</td>
<td>1.97 µl</td>
</tr>
<tr>
<td>Bio-CTP solution(1:10 diluted)</td>
<td>3 µl</td>
</tr>
<tr>
<td>PCR product</td>
<td>150 ng</td>
</tr>
<tr>
<td>Enzyme Mix</td>
<td>2 µl</td>
</tr>
<tr>
<td>NFW</td>
<td>Upto 20 µl</td>
</tr>
</tbody>
</table>
The reaction was incubated at 37°C for 2 h. After incubation, 1 μl of TURBO DNase (Megascript kit component) was mixed and kept at 37°C for 15 min. To the reaction tube, 15 μl of 3M Ammonium acetate was added to the reaction mix and volume was made up to 150 μl with nuclease free water. To the same tube 150 μl of phenol: chloroform mix was added and mixed properly by inverting the tube 10 times. Tubes were spun at 10,000xg for 5 min and supernatant was taken into a fresh tube. To the supernatant equal amount of isopropanol was added and kept in -80°C for 20 min or O/N for RNA precipitation. Tubes were spun at 4°C at 10,000xg for 15 minutes to pellet RNA and pellet was washed with 70% ethanol. RNA was resuspended in nuclease free water for further experiments. For long term storage, 0.01% SDS was added to RNA to prevent its degradation. RNA was run of 1.2% formaldehyde gel for checking the length of probe synthesized.

Lysate preparation

Cells were grown in 5 plates of 100 mm plates up to confluency containing 1 X10^7 cells/plate were washed with 1X ice cold PBS. Cells were scrapped and collected in 15 ml tube. These tubes were spun at 4°C for 5 min at 2000xg to pellet the cells. To the pellet, 3 volumes of IP lysis buffer (Pierce) containing 1X protease inhibitor complex (PIC) (Sigma), was added and vortexed gently. Solution was aliquoted in 1.5 ml tubes and kept on ice for 30 min to allow complete lysis of cells. These tubes were then spun at 4°C, 10,000xg for 20 min. Lysates were aliquote and kept in -80°C for further use. For preparing lysates from 14.5 dpc mouse brain, mouse brain from 14.5 dpc were dissected under dissection microscope and were minced directly into IP lysis buffer containing 1X PIC using gentleMACS dissociator (Miltenyi biotech).

Immuno-precipitation of RNA protein complex.

1 mg of whole cell lysates were incubated for 15 min in 3 fold excess of binding buffer with 10 μl μMACS beads (magnetic beads conjugated with streptavidin) and with 30 μl of 10 μg/μl of yeast tRNA for clearance of non-specific proteins binding to beads and any RNA prior to immunoprecipitation. After 15 min, beads were removed by passing the solution having lysate, beads and tRNA from μ column kept on a magnetic stand. Supernatant was taken in a fresh 15 ml tube and kept on ice until use. Meanwhile 3 μg of RNA was heated at 90°C for 2 min and then quick-chilled on ice. 3 μl of 40 u/μl of RNasin (Promega) and 20 μl of RNA binding buffer was added to it and mixed thoroughly. RNA mix
was incubated at room temperature for 20 min. Also for competition, 60 μg (20X excess) of unlabelled RNA + 3 μg of biotinylated RNA was added in another tube and same treatment was given to it. After, allowing RNA to fold, it was added to precleared lysates and kept for 1 h at 4ºC on a rotating platform to allow RNA to bind proteins. After, RNA-protein complexes are formed 60 μl of μMACS beads were added to the lysate and kept at 4ºC for 15 min on rotating platform. RNA-protein complex by passing the lysate with RNA from μ column as described above. μ Columns, having RNA-protein complex bound beads, were washed four times with 100 μl of binding buffer. RNA-protein complexes were eluted by adding 50 μl of RNA-protein elution buffer to the beads. The elutes were boiled in 1X lamelli buffer for 3-4 minutes and supernatant was loaded onto 10% SDS PAGE gel.

**In gel tryptic digestion for Mass-spectroscopy.**

After running the gel for resolving proteins, it was stained with Comassie Blue stain (Sigma) for 2 h and then kept in destaining solution for over-night. Protein bands seen in the gel were for those proteins, which interacted with RNA. Those bands were excised after capturing the image using Gel-Doc. Excised bands were kept in methanol and distilled water treated 1.5 ml tube and was dehydrated using 100 μl of dehydration solution for 5 min at room temperature. Supernatant was removed from gel slice and 100 μl of 25mM ammonium bicarbonate solution was added for 2 min at room temperature. Dehydration and rehydration step was repeated once. Gel slice was dehydrated by adding 100 μl of dehydration solution for 5 min at room temperature and preceded for reduction and alkylation step. Here the gel pieces were rehydrated using 10mM DTT solution for 2 min and were kept at 60ºC for 1 h. DTT solution was then removed and gel was rinsed with 100 μl 25 mM Ammonium Bicarbonate solution. To the gel piece, we added 50-100 μl of 100 mM iodoacetamide (IAA). Gel slices were incubated at room temperature in the dark for 20 min. IAA was removed and gel slice was senssed with 50 μl Ammonium bicarbonate. Gel slice was again dehydrated using 100 μl dehydration solution for 5 min. Supernatant was removed and 50 μl of 25 mM Ammonium bicarbonate solution was added for 2 min. supernatant was removed and gel slices were dried using speed vac for 30 min.

The gel slice was rehydrated with activated trypsin (100 μl) on ice and incubated on ice 20-30 min until trypsin was absorbed. After gel slice was completely rehydrated, 25mm Ammonium bicarbonate solution was added to
cover gel slice in tube. Tubes were incubated at 37°C for 16 h. Supernatant was removed and gel slice was vortexed with extraction buffer. Extractions were repeated once more and combined with the supernatant. Total volume was reduced using speed vac to 10-20 ul. This solution was used for mass spec after purifying peptides using ZIPTip (Millipore). ZipTip was wetted by aspirating 10 µl wetting solution into tip and dispensing to waste twice. Peptides were bound to ZipTip pipette tip by aspirating and dispensing the solution 10-12 times; bound peptides were washed by aspirating washing solution 5 times. After washing, the bound peptides were dispensed in elution solution, which was taken in a clean 0.5 ml tube using a standard pipette tip. Aspirate and dispense eluant was aspirated at least 10 times to ensure all the peptides were eluted. These samples were given to Mass spectroscopy facility for analyses.

2.16. Derivation of Induced pluripotent cells using Ginir
Reagents and Solutions

1X PBS
Solution was prepared as described in section 2.1.

0.2% gelatine solution
0.2 g of gelatine was dissolved in 100 ml of sterile 1X PBS and was autoclaved for sterilization.

Stock Mitomycin C solution (1 mg/ml)
1 mg of Mitomycin C (Sigma Aldrich) was dissolved completely in 1 ml of deionized autoclaved water. Solution was filter sterilized using 0.22 µm filter, aliquoted and stored in -20°C until use.

Working Mitomycin C solution
10 µl of mitomycin C was diluted in 1 ml of complete medium to prepare a working solution of 10 µg/µl. This solution was always prepared fresh and was directly added to cells.

Methodology
Mouse embryonic fibroblast and SK-N-MC cells transfected with Ginir and Giniras pTargeT vector were dissociated non-enzymatically and were put onto
feeder layers prepared from 14.5 dpc mouse embryos isolated from Swiss Webster (CFW®) impregnated female mice. For feeder layers, head and tail regions of 14.5 dpc mouse embryos were excised and all the visceral organs were removed using dissection forcep under sterile conditions and the remaining tissue was washed with sterile 1X PBS. The tissue was finely minced with a sterile blade and 1 ml of TPVG solution was added to each embryo. Tissue with TPVG solution was transferred to a 50 ml falcon and incubated for 15 min at 37°C in a humidified chamber with 5% CO₂. After every 5 min, cells were dissociated by pipetting solution up and down thoroughly. Trypsin was inactivated by adding equal volume of complete medium. Cells were then centrifuged with low-speed (300xg) for 5 min at room temperature and supernatant was carefully removed and pellet was resuspended in complete medium. Cells were plated onto 0.2% gelatine coated flasks (Gelatine treatment was given for 2 h before plating the cells). Cells were allowed to reach 80-90% confluency and at this stage a major part of passage 0 (P0) cells were frozen for future usage. For feeder preparation, 150 cm² flasks were coated with gelatine and incubated at RT for 2 h. Cells were seeded onto the gelatine coated flasks and 70-80% confluent cells were given Mitomycin C treatment by adding 5 ml of working solution of Mitomycin C for 2 h at 37°C and then washed twice with 1X sterile PBS, trypsinized, centrifuged (for 5 min at 300xg) and resuspend in warm medium. Cells were counted and plated at a density of 5X10⁴ cells per 60 mm dish and were used next day. Ginir and Giniras transfected mouse embryonic fibroblasts and SK-N-MC cells were allowed to reach 80-90% confluency. These cells were dissociated non-enzymatically and were plated onto feeder at day 0 in human embryonic stem cell medium (DMEM nutrient mix F12 supplemented with 20% (v/v) KOSR, 1% (v/v) GlutaMAX, 1% (v/v) NEAA, 1X penicillin and streptomycin, 100 μM β mercaptoethanol and 10 ng ml⁻¹ bFGF) at density of 1 X 10⁴ cells per plate. Medium was changed every alternate day and iPS like colonies appeared after 7 days.

2.17. Alkaline phosphatase staining

Reagents and Solutions

Alkaline phosphatase detection kit was procured from Sigma Aldrich which contained following components

FAST BLUE RR SALT capsules. These capsules were ready to use and were always stored at 4°C.
FAST VIOLET B SALT 12 mg capsules. These capsules were ready to use and were always stored at 4ºC.

NAPHTHOL AS-MX PHOSPHATE ALKALINE SOLUTION (Naphthol AS-MX phosphate, 0.25% (w/v), buffered at pH 8.6, 25ºC). This solution was ready to use and was stored at 4ºC.

MAYER’S HEMATOXYLIN SOLUTION (Hematoxylin, certified, 1 g/l, sodium iodate, 0.2 g/l, aluminum ammonium hydroxide, 5 g/l, and stabilizers.) This solution was ready to use and was stored at room temperature.

CITRATE CONCENTRATED SOLUTION (Contains citric acid-sodium citrate, 1.5 mol/l.)

Citrate working solution
2 ml of Citrate concentrate solution was dissolved in 100 ml with deionized water.

Fixative Solution (citrate buffered acetone, 60%)
This solution was prepared by mixing 2 volumes of citrate working solution with 3 volumes of acetone.

Methodology

1 capsule of FAST Blue RR salt was dissolved in 48 ml distilled water and kept on magnetic stirrer to prepare diazonium salt. To this solution, 2 ml Naphthol AS-MX Phosphate Alkaline Solution was added and mixed thoroughly. Meanwhile, 60 mm Fix plates were washed with 1X PBS and fixed using citrate buffered acetone for 30 seconds. Plates were then rinsed with deionized water for 45 seconds. After that the alkaline-dye mixture prepared above was added to the plates and incubated at 18–26ºC for 30 minutes. After 30 minutes, cells were again washed with deionized water and stained using Mayer’s Hematoxylin Solution for 10 minutes. Images of these slides were captured in bright-field using BX61 upright microscope (Olympus).

2.18. Immuno-precipitation assay

Reagents and Solutions

1X PBS
Solution was prepared as described in section 2.1.
Methodology

For performing immuno-precipitation of cyclin D1 and cyclin E, cells from 2 X10 cm plates were washed and scraped in chilled 1X PBS. These cells were spun at 1000xg, 4°C for 5 min and to it IP lysis buffer (Pierce) containing Protease inhibitor cocktail (Sigma) was added. For immunoblotting, 2 mg of total protein was taken and was precleared by adding 20 μl of magnetically labelled protein A/G beads (Invitrogen) for 30 min. After that, beads bound to non-specific proteins were separated by keeping the tubes on magnetic stand for at least 1 min. For immunoprecipitations, 2 μg antibodies for cyclin D1 and cyclin E were allowed to bind to their respective proteins and complexes for 3 h to over-night at 4°C. The protein complexes were pulled down by addition of magnetically labelled protein A/G beads for 1 h. After, 1 h beads were collected by keeping the tubes on magnetic stand for at least 1 min and were thoroughly washed with IP-lysis buffer. After washing, beads were directly heated in 1X lamelli buffer for 3-4 min at 94°C. Beads were again separated on magnetic stand and supernatant was loaded on 12% SDS-PAGE gel. The gel was allowed to run and was transferred onto PVDF membrane as described in section 2.13. Blots obtained after immuno-precipitation of cyclin D1 and cyclin E were assayed for their associated proteins, cdk-4 and cdk-2, respectively.